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Association Studies and Direct DNA Sequencing Implicate Genetic Susceptibility Loci in the Etiology of Nonsyndromic Orofacial Clefts in Sub-Saharan African Populations

L.J.J. Gowans[1,2,3,4*], W.L. Adeyemo[5*], M. Eshete[6*], P.A. Mossey[7], T. Busch[4], B. Aregbesola[8], P. Donkor[2,9], F.K.N. Arthur[1], S.A. Bello[10], A. Martinez[4], M. Li[4], E.A. Augustine-Akpan[4], W. Deressa[6], P. Twumasi[1], J. Olutayo[5], M. Deribew[6], P. Agbenorku[2,9], A.A. Oti[2,9], R. Braimah[8], G. Plange-Rhule[2], M. Gesses[11], S. Obiri-Yeboah[2,9], G.O. Oseni[12], P.B. Olaitan[12], L. Abdur-Rahman[13], F. Abate[11], T. Hailu[11], P. Gravem[14], M.O. Ogunlewe[12], C.J. Buxó[15], M.L. Marazita[16], A.A. Adeyemo[17], J.C. Murray[3], and A. Butali[4]

Abstract

Orofacial clefts (OFCs) are congenital dysmorphologies of the human face and oral cavity, with a global incidence of 1 per 700 live births. These anomalies exhibit a multifactorial pattern of inheritance, with genetic and environmental factors both playing crucial roles. Many loci have been implicated in the etiology of nonsyndromic cleft lip with or without cleft palate (NSCL/P) in populations of Asian and European ancestries, through genome-wide association studies and candidate gene studies. However, few populations of African descent have been studied to date. Here, the authors show evidence of an association of some loci with NSCL/P and nonsyndromic cleft palate only (NSCPO) in cohorts from Africa (Ghana, Ethiopia, and Nigeria). The authors genotyped 48 single-nucleotide polymorphisms that were selected from previous genome-wide association studies and candidate gene studies. These markers were successfully genotyped on 701 NSCL/P and 163 NSCPO cases, 1,070 unaffected relatives, and 1,078 unrelated controls. The authors also directly sequenced 7 genes in 184 nonsyndromic OFC (NSOFC) cases and 96 controls from Ghana. Population-specific associations were observed in the case-control analyses of the subpopulations, with West African subpopulations (Ghana and Nigeria) showing a similar pattern of associations. In meta-analyses of the case-control cohort, *PAX7* (rs742071, $P = 5.10 \times 10^{-3}$), 8q24 (rs987525, $P = 1.22 \times 10^{-3}$), and *VAX1* (rs7078160, $P = 0.04$) were nominally associated with NSCL/P, and *MSX1* (rs115200552, $P = 0.01$), *TULP4* (rs651333, $P = 0.04$), *CRISPLD2* (rs4783099, $P = 0.02$), and *NOG1* (rs17760296, $P = 0.04$) were nominally associated with NSCPO. Moreover, 7 loci exhibited evidence of threshold overtransmission in NSOFC cases through the transmission disequilibrium test and through analyses of the family-based association for disease traits. Through DNA sequencing, the authors also identified 2 novel, rare, potentially pathogenic variants (p.Asn323Asp and p.Lys426IlefsTer6) in *ARHGAP29*. In conclusion, the authors have shown evidence for the association of many loci with NSCL/P and NSCPO. To the best of this knowledge, this study is the first to demonstrate any of these association signals in any African population.

Keywords: genetic heterogeneity, rare variants, genome-wide association studies (GWAS), candidate genes, craniofacial genetics, population genetics

Introduction

Human orofacial clefts (OFCs) are congenital malformations of the face and oral cavity due to dysregulation of embryologic processes. The global incidence of OFCs is 1 per 700 live births. However, race, ethnicity, geographic locations, environmental factors, and socioeconomic status influence the incidence of OFCs (Gorlin et al. 2001). The highest incidence occurs in Asians, followed by populations of European ancestry, whereas African populations have the lowest incidence (Mossey and Modell 2012). Although there are no national prevalence data for Ghana and Ethiopia, an estimate of 0.5 per 1,000 has been observed for Nigeria (Butali, Adeyemo, et al. 2014). These observations presuppose that the relative contributions of individual susceptibility genes may vary across different human populations. OFCs may be syndromic or nonsyndromic, with the syndromic forms presenting with other congenital anomalies. The etiology of the more common nonsyndromic OFCs (NSOFCs) is complex, exhibiting multifactorial pattern of inheritance. NSOFCs are classified into nonsyndromic cleft lip with or without cleft palate (NSCL/P) and nonsyndromic cleft palate only (NSCPO), and these 2

groups have a heterogeneous genetic architecture. NSCL/P comprises nonsyndromic cleft lip only (NSCL) and nonsyndromic cleft lip and palate (NSCLP; Dixon et al. 2011).

To date, 6 genome-wide association studies (GWASs) and a meta-analysis have been published for NSOFCs, with these signals demonstrating an association with NSCL/P but not NSCPO. In a GWAS involving Europeans, an association was observed between a locus in Chr8q.24 and NSCL/P (Birnbaum et al. 2009). The 8q.24 signal was subsequently replicated in another GWAS of NSCL/P in Europeans from the United States (Grant et al. 2009). A third GWAS that involved cohorts of European ancestries also revealed that 2 additional loci, 17q22 (*NOG1*) and 10q25 (*VAX1*), were associated with NSCL/P. Other loci yielded a suggestive association with NSCL/P: 15q13.3 (*GREM1*), 13q31.1 (*SPRY2*), and 2p21 (*THADA*; Mangold et al. 2010). Employing trios of Asian and European ancestries, a GWAS implicated 20q12 (*MAFB*) and 1p22.1 (*ABCA4*) in the etiology of NSCL/P, with 17p13 (*NTN1*) showing a suggestive association. Stratified analyses based on ancestries by the same GWAS showed that some signals were ancestry specific: trios of European ancestry gave the strongest association for 8q.24, whereas those of Asian ancestry were strongly associated with *MAFB, ABCA4*, and *IRF6* (Beaty et al. 2010). A meta-analysis revealed additional NSCL/P susceptibility loci: *THADA, SPRY2*, 15q22.2 (*TPM1*), and 1p36 (*PAX7*; Ludwig et al. 2012). Recently, a GWAS involving Asians implicated 16p13.3 (*ADCY9*; Sun et al. 2015) in the etiology of NSCL/P, whereas a GWAS involving dogs and a Guatemalan population gave a suggestive association for *ADAMTS20* (Wolf et al. 2015).

In the pre- and post-GWAS era, candidate gene and replication studies have been instrumental in identifying cleft susceptibility loci. Pathogenic variants in *IRF6* were shown to cause van der Woude syndrome and popliteal pterygium syndrome (Kondo et al. 2002). Subsequently, a missense variant in *IRF6* (rs2235371) demonstrated overtransmission in NSCL/P cases of European ancestry (Zucchero et al. 2004). Another *IRF6* locus, rs642961, has been shown to be associated with NSCL/P but not NSCPO (Rahimov et al. 2008). Corollary to these observations, some studies (Birnbaum et al. 2009; Kerameddin et al. 2015) have confirmed a role of *IRF6* as a NSCL/P risk locus in populations of Asian and European ancestries. Other candidate genes implicated in the etiology of NSCL/P included *MSX1* (Rafighdoost et al. 2013), *BMP4* (Suzuki et al. 2009), *FOXE1* (Moreno et al. 2009), *AXIN2* (Letra et al. 2012), *CRISPLD2* (Chiquet et al. 2007), *NOG1*, and *FGFR2* (Leslie et al. 2015).

Among Africans, genetic studies on OFCs are limited. A study involving a Nigerian cohort implicated *MSX1*, but not other loci, in the etiology of NSCL/P (Butali et al. 2011). Other studies that recruited Kenyans (Weatherley-White et al. 2011) and Congolese (Figueiredo et al. 2014) could not replicate the association for cleft susceptibility loci among Africans, probably due to the small sample size and population heterogeneity. Moreover, sequencing of GWAS loci in cohorts from Ethiopia and Nigeria reported some rare, potentially causative variants (Butali, Mossey, et al. 2014). Conducting genetic and genomics studies with a cleft cohort from Africa may identify novel and population-specific signals. However, it is also important for us to investigate the role of identified signals and biologically relevant genes from existing European and Asian studies in the African population. The present study aimed to replicate the association between reported GWASs and candidate gene loci in our NSCL/P cohort. We also tested the hypothesis that NSCL/P loci may contribute to NSCPO susceptibility in Africans. Finally, we screened for rare, potentially pathogenic variants in 7 candidate genes at risk loci usually associated with NSCL/P.

[1]Department of Biochemistry and Biotechnology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana
[2]Cleft Clinic, Komfo Anokye Teaching Hospital, Kumasi, Ghana
[3]Department of Pediatrics, University of Iowa, Iowa City, IA, USA
[4]Department of Oral Pathology, Radiology and Medicine, University of Iowa, Iowa City, IA, USA
[5]College of Medicine, University of Lagos, Lagos, Nigeria
[6]Addis Ababa University, Addis Ababa, Ethiopia
[7]Department of Orthodontics, University of Dundee, Dundee, Scotland
[8]Obafemi Awolowo University Teaching Hospital, Ile-Ife, Nigeria
[9]Department of Surgery, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana
[10]Department of Oral and Maxillofacial Surgery, State House Hospital, Abuja, Nigeria
[11]Yekatit 12 Hospital Medical College, Addis Ababa, Ethiopia
[12]Department of Burns and Plastic Surgery, Ladoke Akintola University of Technology Teaching Hospital, Osogbo, Nigeria
[13]Division of Pediatric Surgery, Department of Surgery, University of Ilorin, Ilorin, Nigeria
[14]Haukeland University Hospital Bergen, Bergen, Norway
[15]School of Dental Medicine, University of Puerto Rico Medical Science Campus, San Juan, Puerto Rico
[16]Department of Oral Biology, University of Pittsburgh, Pittsburgh, PA, USA
[17]Center for Research on Genomics and Global Health, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA
*Authors contributing equally to this article

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Corresponding Author:
A. Butali, Department of Oral Pathology, Radiology and Medicine/Dows Institute for Dental Research, College of Dentistry, University of Iowa, Butali Laboratory, ML2198, 500 Newton Road, Iowa City, IA 52242, USA.
Email: azeez-butali@uiowa.edu

Subjects and Methods

We recruited 3,585 participants from Ghana, Ethiopia, and Nigeria (Table 1; Appendix Methods). All sample and data collection at various study sites were approved by the local institutional review boards: College of Health Sciences, KNUST (Ghana; CHRPE/AP/217/13); College of Medicine, University of Lagos (Nigeria; ADM/DCST/HREC/APP/1374); and College of Health Sciences, Addis Ababa University (Ethiopia; 3.10/027/2015). Before sample and data collection, written informed consent was obtained from each participating family. DNA processing is shown in the Appendix Methods.

Single-Nucleotide Polymorphism Selection

We selected single-nucleotide polymorphisms (SNPs) with a minor allele frequency (MAF) ≥5% in the African population for genotyping; these were previously reported in peer review journals or identified in animal studies and during our resequencing studies. These include SNPs that are associated with NSCL/P in candidate genes studies and GWASs in European and Asian populations (Appendix Table 1).

SNP Genotyping

We genotyped 48 SNPs (Appendix Table 1) on a total of 3,585 samples—872 NSOFC cases (163 NSCPO, 340 NSCL, 361 NSCLP, and 8 "untyped"), 1,635 unaffected relatives, and 1,078 unrelated controls—with the 192.24 Fluidigm SNP genotyping protocol (Appendix Methods). The "untyped" samples (from probands) and other samples, however, failed quality control checks and were not included in the final statistical analyses (Table 1).

Statistical Analyses for Association Studies

During quality control checks, we resolved Mendelian errors in case-parent triads and dropped from the final analyses samples that were not successfully genotyped on at least 95% of the 48 genotyped SNPs. We computed Hardy-Weinberg equilibrium (HWE) through PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/). We then conducted 1) case-control analyses to determine associations in each subpopulation and 2) meta-analyses of the 3 subpopulations based on Table 1. For this test, we used $P < 0.05$ to denote nominal association and a Bonferroni correction of 141 tests to ascertain a threshold for formal significance of $P = 3.54 \times 10^{-4}$. The 141 tests comprised 47 SNPs that passed HWE × 3 cleft subphenotypes × 1 racial group × 1 test. Of the 48 SNPs, only 1 failed HWE ($P < 0.05$). Additional analyses to determine overtransmission of the rare alleles were conducted with the transmission disequilibrium test (TDT) and through the family-based association for disease traits (DFAM). The TDT used only the case-parent triad information (Table 1), while the DFAM allowed us to combine triad and dyad data. For these tests, the significant *P* value was 0.05. Parent-of-origin effects and gene-gene interactions (epistasis) were also calculated. The probands in the case-control arm of the study (Table 1) are the same probands in the family-based studies.

Table 1. Subphenotypes, Sex, and Sample Types of Study Cohort That Passed Quality Control Checks and Were Included in Statistical Analyses.

Cleft Subphenotype of Probands	Samples per Population, *n*			
	Ghana	Ethiopia	Nigeria	Total
Case-control cohort				
NSCL	162	101	77	340
NSCLP	144	143	74	361
NSCPO	102	21	40	163
Unrelated controls	408	357	313	1078
Case-parent trios				
NSCL	52	2	20	74
NSCLP	48	3	26	77
NSCPO	34	1	7	42
Case-parent dyads				
NSCL	77	84	51	212
NSCLP	76	134	47	257
NSCPO	53	20	32	105
Other trios				
NSCL	18	0	0	18
NSCLP	14	0	0	14
NSCPO	11	0	0	11
Other dyads				
NSCL	8	0	0	8
NSCLP	3	0	0	3
NSCPO	3	0	0	3
Singletons				
NSCL	5	13	6	24
NSCLP	1	8	1	10
NSCPO	2	0	1	3
Tetrads				
NSCLP	2	0	0	2
Pentads				
NSCLP	1	0	0	1

Case probands consisted of 423 males and 441 females, whereas unrelated controls were made up of 441 males and 637 females. The probands in the case-control arm of the study are the same probands in the family-based studies. In some of the designated singletons, parental samples failed data cleaning and were dropped from statistical analyses—hence, the designation of such families as *singletons*. Singletons were informative in the case-control arm of our study but not the family-based studies. Tetrads and pentads were collected from families where 2 individuals were affected with clefts. "Other trios and dyads" largely refers to case-mother-maternal grandmother trios, case-mother-sibling trios, as well as case-siblings trios and dyads. Case-parent trios, tetrads, and pentads were employed in the transmission disequilibrium test, whereas all sample types, except singletons and unrelated controls, were used for analyses of the family-based association for disease traits. Only case probands and unrelated controls were included in the case-control analyses.
NSCL, nonsyndromic cleft lip; NSCL/P, nonsyndromic cleft lip with or without cleft palate; NSCLP, nonsyndromic cleft lip and palate; NSCPO, nonsyndromic cleft palate only.

DNA Sequencing

We directly sequenced *VAX1, PAX7, ARHGAP29, MSX1, FOXE1, BMP4*, and *MAFB* in 184 NSOFC cases (131 NSCL/P and 53 NSCPO) from Ghana using Sanger Sequencing (Appendix Methods; Butali, Mossey, et al. 2014). We also performed segregation

analyses on observed potentially pathogenic missense, frameshift, and splice site variants by sequencing available parental samples. We further sequenced 96 unrelated Ghanaian controls to ascertain whether the novel variants that we encountered in NSOFC cases also occurred in controls.

Results

Association Analyses

In meta-analyses of the case-control cohorts from the 3 subpopulations, we successfully demonstrated nominal association of *PAX7* (rs742071, $P = 5.10 \times 10^{-3}$), 8q24 (rs987525, $P = 1.22 \times 10^{-3}$), as well as *VAX1* (rs7078160, $P = 0.04$) with NSCL/P; in addition, *MSX1* (rs115200552, $P = 0.01$), *TULP4* (rs651333, $P = 0.04$), *CRISPLD2* (rs4783099, $P = 0.02$), and *NOG1* (rs17760296, $P = 0.04$) were nominally associated with NSCPO (Table 2), with the direction of effect being the same as reported by earlier studies. Among Ethiopians (Appendix Table 2), *PAX7* (rs742071, $P = 5.57 \times 10^{-3}$), *IRF6* (rs642961, $P = 0.02$), *DYSF* (rs2303596, $P = 2.31 \times 10^{-3}$), 8q24 (rs987525, $P = 7.82 \times 10^{-4}$), and *MAFB* (rs13041247 and rs11696257, all with $P = 0.04$) were nominally associated with NSCL/P; *ABCA4* (rs481931 and rs4147811, all with $P = 0.03$) and *NTN1* (rs8081823, $P = 0.03$) were nominally associated with NSCPO. Moreover, subphenotype analyses of the Ethiopian NSCL/P cohort showed that the *PAX7, DYSF, MSX1, SPRY2* (rs9574565, $P = 7.05 \times 10^{-3}$) and *MAFB* signals were particularly stronger for NSCL, whereas the *IRF6* (rs642961, $P = 9.11 \times 10^{-3}$) and 8q24 (rs987525, $P = 1.07 \times 10^{-3}$) signals were stronger for NSCLP (Appendix Table 2). Among Ghanaians (Appendix Table 3), *ABCA4* (rs560426, $P = 0.03$) and *VAX1* (rs7078160, $P = 0.03$) were nominally associated with NSCL/P with subphenotype analyses of the NSCL/P cohort showing that the *ABCA4* locus was strongly associated with NSCLP. *ABCA4* (rs4147811, $P = 7.48 \times 10^{-3}$) and *CRISPLD2* (rs4783099, $P = 0.04$) were nominally associated with NSCL/P and NSCPO, respectively, among Nigerians (Appendix Table 4). Subphenotype analyses of the Nigerian NSCL/P (Appendix Table 4) showed that *PAX7* (rs742071, $P = 0.02$) and *ARHGAP29* (rs138751793, $P = 0.04$) signals were stronger for NSCL, whereas another SNP at the *ABCA4* locus (rs481931, $P = 2.87 \times 10^{-3}$) was strongly associated with NSCLP. However, none of these case-control associations passed Bonferroni correction.

For the TDT and DFAM (Tables 3 and 4) for all 3 subpopulations, 7 loci demonstrated formal significance with NSOFCs at $P \leq 0.05$. Formal significance for the TDT and DFAM was evaluated at $P \leq 0.05$ because these are secondary analyses compared with case-control analyses and are not true independent tests. All family-based studies suggested that the minor allele of *ABCA4* (rs560426) was overtransmitted in NSCLP cases among Africans. *PAX7* (rs742071) also consistently showed evidence of overtransmission in NSCL cases in the TDT and DFAM. *MSX1* (rs115200552) and *AXIN2* (rs3923086) also demonstrated strong overtransmission in NSCLP cases in DFAM analyses, whereas *MTHFR* (rs1801131) and *DYSF* exhibited overtransmission in NSCL cases in TDT and DFAM analyses, respectively. Only an SNP of *VAX1* demonstrated overtransmission in NSCPO cases.

Parent-of-Origin Effects

Parent-of-origin effects were not observed for almost all SNPs, except rs16260 of *CDH1*. For rs16260, a trend toward association ($P = 0.0764$) was observed for all clefts. The rs16260 SNP exhibited a maternal imprinting or maternal overtransmission effect.

Gene-Gene Interactions

In gene-gene (G × G) or epistatic interactions, 3 SNPs exhibited evidence of epistasis with other SNPs. Each of these epistatic interactions yielded $P = 0.02$. A SNP for *ABCA4*, rs560426, interacted with *Chr6*, rs2674394 (gene desert). Moreover, rs2303596 of *DYSF* interacted with rs3923086 of *AXIN2*. Finally, rs8069536 of *NTN1* interacted with rs17820943, rs13041247, and rs11696257, all of *MAFB*. However, none of these G × G interactions passed Bonferroni correction.

Direct DNA Sequencing of 7 Selected Genes

We observed several rare and/or novel variants in the 7 genes that we sequenced (Table 5, Appendix Table 5). "Rare variants," as used here, refer to either a novel variant or a variant whose MAF is ≤1%. Some of these variants were predicted to be potentially pathogenic by various bioinformatics tools, whereas others were depicted as benign. A de novo occurrence could not be demonstrated for any of these variants, because either the variant was present in at least 1 parent, or not both parents were available for segregation analysis. Last, some of the novel variants that we observed occurred in controls (e.g., all *VAX1* variants), whereas others were not observed in controls (e.g., all *ARHGAP29* variants).

Discussion

We have successfully demonstrated associations (both nominal in case-control analyses and threshold in the TDT and DFAM analyses) between some loci and NSCL/P in cohorts from Africa. We also tested the hypothesis that these loci contribute to NSCPO in Africans, and we observed some interesting associations. The 8q24 locus exhibited the strongest nominal significance with NSCL/P in case-control meta-analyses, with the trends suggesting that this locus may be relevant in all 3 subpopulations. The test of heterogeneity also largely suggested the absence of heterogeneity at this locus among the 3 African populations. We observed that among Africans, the associated minor C allele of rs987525 (http://browser.1000genomes.org) conferred reduced susceptibility, while the major A allele is the risk allele. Irrespective of these differences in minor alleles, our result is in harmony with earlier

Table 2. Meta-analyses of the Case-Control Cohorts from Ghana, Ethiopia, and Nigeria.

Part A: Meta-analyses of NSCL/P and NSCPO Case-Control Cohorts from All 3 Countries

				NSCL/P			NSCPO		
SNP	Probable Gene/Loci	Minor Alleles[a]	African MAF	*P*	OR	I	*P*	OR	I
rs1801131	*MTHFR*	C/A[b]	0.15	0.32	1.08	0.00	0.19	0.79	0.00
rs1801133	*MTHFR*	A/G[c]	0.09	0.49	1.08	18.19	0.44	0.83	0.00
rs766325	*PAX7*	G/A[b,d,e]	0.18	0.29	0.92	0.00	0.23	0.82	0.00
rs742071	*PAX7*	T/G[b]	0.39	**5.10E-03**[f]	**1.19**	**54.68**	0.76	0.96	0.00
rs560426	*ABCA4*	C/T[b,g]	0.49	0.10	0.90	6.15	0.16	1.18	0.00
rs481931	*ABCA4*	T/G[c]	0.10	0.40	1.09	11.13	0.49	0.85	0.00
rs4147811	*ABCA4*	T/C[c]	0.11	0.23	1.13	67.35	0.93	1.02	0.00
rs138751793	*ARHGAP29*	C/T[h]	0.02	0.24	1.32	0.00	0.47	1.34	27.90
rs6677101	*SLC25A24*	G/T[b,e,g]	0.33	0.80	0.98	12.11	0.87	1.02	53.89
rs861020	*IRF6*	A/G[b]	0.11	0.23	1.11	0.00	0.83	0.96	24.15
rs34743335	*IRF6*	T/A	0.02	0.59	0.90	0.00	0.84	0.89	38.34
rs642961	*IRF6*	A/G[b]	0.09	0.32	1.11	68.47	0.57	0.88	44.17
rs7590268	*THADA*	G/T[b]	0.20	0.74	0.98	0.00	0.38	0.87	0.00
rs4332945	*DYSF*	T/G[b,e,g]	0.16	0.94	0.99	0.00	0.97	1.01	0.00
rs2303596	*DYSF*	T/C[c,d,e]	0.22	0.20	0.91	75.32	0.57	1.09	73.54
rs227782	*DYSF*	A/G[b,g]	0.42	0.33	1.06	0.00	0.35	1.12	61.90
rs115200552	*MSX1*	C/G[h]	0.02	0.38	1.16	28.63	**0.01**[f]	**1.81**	**0.00**
rs12532	*MSX1*	G/A[c,e]	0.44	0.49	0.96	0.00	0.37	0.90	0.43
rs2674394	Gene desert	A/C[b]	0.17	0.62	1.04	0.00	0.68	1.07	0.00
rs651333	*TULP4*	C/T[b,d,g]	0.34	0.97	1.00	0.00	**0.04**[f]	**1.29**	**0.00**
rs6558002	*EPHX2*	C/T[b,g]	0.24	0.39	1.06	0.00	0.87	1.02	0.00
rs987525	8q24	A/C[b,g]	0.38	**1.22E-03**[f]	**0.81**	**40.55**	0.22	0.86	0.00
rs894673	*FOXE1*	A/T[c]	0.33	0.42	0.95	0.00	0.93	1.01	0.00
rs3758249	*FOXE1*	T/C[c]	0.33	0.56	0.96	0.00	0.90	1.02	0.00
rs7078160	*VAX1*	A/G[b]	0.25	**0.04**[f]	**1.16**	**0.00**	0.88	1.02	0.00
rs4752028	*VAX1*	C/T[b,g]	0.45	0.51	0.96	0.00	0.80	0.97	0.00
rs10785430	*ADAMTS20*	G/A[b]	0.32	0.90	0.99	0.00	0.49	1.09	0.00
rs9574565	*SPRY2*	T/C[c,g]	0.35	0.75	1.02	0.00	0.45	1.10	0.00
rs8001641	*SPRY2*	G/A[c,d,e,g]	0.10	0.35	1.08	0.00	0.37	0.85	0.00
rs17563	*BMP4*	T/C[b,d,e,g]	0.18	0.95	0.99	0.00	0.77	1.04	0.00
rs1258763	*GREM1*	C/T[c,d,e,g]	0.49	0.11	1.11	0.00	0.50	0.92	0.00
rs8049367	*ADCY9*	C/T[c,d,e]	0.30	0.20	1.09	0.00	0.10	0.81	0.00
rs16260	*CDH1*	A/C[b]	0.13	0.59	1.05	0.00	0.39	0.85	0.00
rs11642413	*CDH1*	G/A[b,e,g]	0.28	0.83	1.02	0.00	0.21	0.83	0.00
rs1546124	*CRISPLD2*	G/C[b,e]	0.25	0.60	0.96	0.00	0.89	0.98	0.00
rs4783099	*CRISPLD2*	T/C[b]	0.33	0.59	1.04	0.00	**0.02**[f]	**0.74**	**0.00**
rs8069536	*NTN1*	T/G[b]	0.32	0.13	1.11	0.97	0.88	0.98	0.00
rs8081823	*NTN1*	A/G[c]	0.24	0.08	0.88	0.00	0.63	0.94	32.54
rs17760296	*NOG1*	G/T[b]	0.02	0.92	0.99	0.00	**0.04**[f]	**1.74**	**0.00**
rs227731	*NOG1*	G/T[b,g]	0.22	0.86	0.99	0.00	0.26	1.17	0.00
rs7224837	*AXIN2*	G/A[b]	0.11	0.75	1.04	0.00	0.81	0.95	0.00
rs3923086	*AXIN2*	A/C[b,d,e,g]	0.02	0.25	1.15	0.00	NA	NA	NA
rs17820943	*MAFB*	T/C[c]	0.25	0.33	0.93	15.15	0.68	1.06	22.99
rs13041247	*MAFB*	C/T[c]	0.25	0.37	0.94	34.01	0.42	1.12	0.00
rs11696257	*MAFB*	T/C[c]	0.25	0.30	0.93	32.24	0.61	1.07	0.00

Part B: Meta-analyses of Subphenotypes of NSCL/P Cohorts from the 3 Countries

				NSCL			NSCLP		
rs1801131	*MTHFR*	C/A[b]	0.15	0.78	1.03	0.00	0.22	1.13	0.00
rs1801133	*MTHFR*	A/G[c]	0.09	0.71	1.06	8.24	0.30	0.30	0.00
rs766325	*PAX7*	G/A[b,d,e]	0.18	0.91	0.99	0.00	0.17	0.86	0.00
rs742071	*PAX7*	T/G[b]	0.39	**0.02**[f]	**1.23**	**68.74**	**0.03**[f]	**1.19**	**0.00**
rs560426	*ABCA4*	C/T[b]	0.49	0.73	1.03	0.00	**0.03**[f]	**1.20**	**10.33**
rs481931	*ABCA4*	T/G[c]	0.10	0.81	0.97	0.00	0.08	1.27	63.75
rs4147811	*ABCA4*	T/C[c]	0.11	0.50	1.10	65.82	0.15	1.21	15.35
rs138751793	*ARHGAP29*	C/T[h]	0.02	0.19	1.53	66.38	0.41	1.29	0.00
rs6677101	*SLC25A24*	G/T[b,e,g]	0.33	0.92	0.99	0.00	0.98	1.00	58.97
rs861020	*IRF6*	A/G[b]	0.11	0.18	1.17	17.72	0.57	1.07	0.00
rs34743335	*IRF6*	T/A	0.02	0.87	0.96	0.00	0.50	0.85	23.72
rs642961	*IRF6*	A/G[b]	0.09	0.96	0.99	15.60	0.15	1.21	62.97
rs7590268	*THADA*	G/T[b]	0.20	0.45	0.92	0.00	0.50	1.07	0.00

(continued)

Table 2. (continued)

Part B: Meta-analyses of Subphenotypes of NSCL/P Cohorts from the 3 Countries									
				NSCL			NSCLP		
SNP	Probable Gene/Loci	Minor Alleles[a]	African MAF	*P*	OR	I	*P*	OR	I
rs4332945	*DYSF*	T/G[b,e,g]	0.16	0.54	0.94	10.40	0.71	1.04	0.00
rs2303596	*DYSF*	T/C[c,d,e]	0.22	0.29	0.89	63.58	0.44	0.93	75.54
rs227782	*DYSF*	A/G[b,g]	0.42	0.85	0.98	0.00	0.13	1.14	0.00
rs115200552	*MSX1*	C/G[h]	0.02	0.18	1.37	61.30	0.68	1.10	0.00
rs12532	*MSX1*	G/A[c,e]	0.44	0.55	0.95	0.00	0.51	0.95	0.00
rs2674394	Gene desert	A/C[b]	0.17	0.06	1.22	0.00	0.42	0.91	0.00
rs651333	*TULP4*	C/T[b,d,g]	0.34	0.63	0.96	0.00	0.74	0.97	0.00
rs6558002	*EPHX2*	C/T[b,g]	0.24	0.82	1.02	0.00	0.11	0.11	0.00
rs987525	8q24	A/C[b,g]	0.38	**5.38E-03**[f]	**1.28**	**0.00**	**0.01**[f]	**0.80**	**54.21**
rs894673	*FOXE1*	A/T[c]	0.33	0.54	0.95	42.39	0.45	0.94	0.00
rs3758249	*FOXE1*	T/C[c]	0.33	0.53	0.94	46.73	0.68	0.96	0.00
rs7078160	*VAX1*	A/G[b]	0.25	**0.03**[f]	**1.23**	**0.00**	0.20	1.13	24.04
rs4752028	*VAX1*	C/T[b,g]	0.45	0.55	1.05	16.64	0.50	0.95	0.00
rs10785430	*ADAMTS20*	G/A[b]	0.32	0.88	1.01	41.30	0.86	0.98	3.00
rs9574565	*SPRY2*	T/C[c,g]	0.35	0.53	1.06	72.62	0.43	1.07	65.44
rs8001641	*SPRY2*	G/A[c,d,e,g]	0.10	0.99	1.00	0.00	0.26	1.13	0.00
rs17563	*BMP4*	A/G[b,d,e,g]	0.18	0.89	0.99	25.84	0.98	1.00	0.00
rs1258763	*GREM1*	C/T[c,d,e,g]	0.49	0.22	0.90	0.00	0.10	1.15	0.00
rs8049367	*ADCY9*	C/T[c,d,e]	0.30	0.36	1.09	10.19	0.35	1.08	0.00
rs16260	*CDH1*	A/C[b]	0.13	0.46	0.91	10.51	0.20	1.16	0.00
rs11642413	*CDH1*	G/A[b,e,g]	0.28	0.98	1.00	0.00	0.55	1.05	0.00
rs1546124	*CRISPLD2*	G/C[b,e]	0.25	0.26	0.90	0.00	0.88	1.01	0.00
rs4783099	*CRISPLD2*	T/C[b]	0.33	0.85	1.02	0.00	0.32	1.09	0.00
rs8069536	*NTN1*	T/G[b]	0.32	0.72	1.03	3.47	**0.04**[f]	**1.20**	**0.00**
rs8081823	*NTN1*	A/G[c]	0.24	0.55	0.95	0.00	0.05	0.83	0.00
rs17760296	*NOG1*	G/T[b]	0.02	0.83	1.04	5.85	0.85	0.97	0.00
rs227731	*NOG1*	G/T[b,g]	0.22	0.38	0.92	0.00	0.59	1.05	0.00
rs7224837	*AXIN2*	G/A[b]	0.11	0.61	1.08	0.00	0.81	1.04	0.00
rs3923086	*AXIN2*	A/C[b,d,e,g]	0.02	0.62	1.10	40.28	NA	NA	0.00
rs17820943	*MAFB*	T/C[c]	0.25	0.25	0.89	15.55	0.43	0.93	0.00
rs13041247	*MAFB*	C/T[c]	0.25	0.25	0.89	31.03	0.54	0.94	0.00
rs11696257	*MAFB*	T/C[c]	0.25	0.24	0.89	27.17	0.40	0.92	0.00

All *P* values reported are for the minor alleles. All initial studies were carried out in Asians and/or Caucasians but not Africans. Source of minor alleles and MAF: http://browser.1000genomes.org.

I, test of heterogeneity of which 0 to 40 represents no heterogeneity; MAF, minor allele frequency; NA, not applicable; NSCL, nonsyndromic cleft lip; NSCL/P, nonsyndromic cleft lip with or without cleft palate; NSCLP, nonsyndromic cleft lip and palate; NSCPO, nonsyndromic cleft palate only; OR, odds ratio; SNP, single-nucleotide polymorphism.

[a]The first allele is the minor allele in Europeans unless otherwise indicated. The first allele is also the minor allele in East Asians, South Asians, and Africans.

[b]Minor allele was the risk allele in initial study.

[c]Minor allele was protective in initial study.

[d]The first allele is the major allele, while the second allele is the minor allele in South Asians.

[e]The first allele is the major allele ,while the second allele is the minor allele in East Asians.

[f]Loci that reached nominal significance in meta-analyses (in bold).

[g]The first allele is the major allele, while the second allele is the minor allele in Africans.

[h]The first allele is the minor allele, and the variation exists only in Africans.

studies (Birnbaum et al. 2009; Grant et al. 2009; Mangold et al. 2010; Beaty et al. 2010; Ludwig et al. 2012) demonstrating that the A allele of rs987525 is a risk allele for NSCL/P in Europeans. These observations suggest that the actual risk variant is (or variants are) in linkage disequilibrium with the A allele of rs987525. Fine mapping of the African haplotype (which is smaller in the 8q24 region) will help identify the risk variant (or variants). Our observations corroborate those made elsewhere (Beaty et al. 2010; Murray et al. 2012) suggesting that the varied ethnic association of the rs987525 allele largely depends on its MAF in various populations. Current evidence suggests that although the 8q24 window is a gene desert, it harbors very remote *cis*-acting craniofacial enhancer elements that regulate the expression of oncogenic *MYC* in the developing face; perturbation of this regulatory network leads to craniofacial dysmorphologies, including sporadic CL/P, in mice (Uslu et al. 2014).

The C677T (rs1801133) SNP of *MTHFR* but not A1298C (rs1801131) has largely been associated with reduced risk for NSCL/P in Asians (Zhao et al. 2014; Martinelli et al. 2015; Pan

Table 3. Transmission Disequilibrium Test for Case-Parent Trios Only.

Part A: Transmission Disequilibrium Test Analyses for NSCL/P and NSCPO

		NSCL/P			NSCPO		
SNP	Probable Gene/Loci	T:NT	*P*	OR (95% CI)	T:NT	*P*	OR (95% CI)
rs1801131	*MTHFR*	27:34	0.37	0.79 (0.48 to 1.32)	10:9	0.82	1.11 (0.45 to 2.73)
rs1801133	*MTHFR*	22:23	0.88	0.96 (0.53 to 1.72)	6:8	0.59	0.75 (0.26 to 2.16)
rs766325	*PAX7*	43:52	0.36	0.83 (0.55 to 1.24)	11:11	1.00	1.00 (0.43 to 2.31)
rs742071	*PAX7*	82:75	0.58	1.09 (0.80 to 1.50)	16:11	0.34	1.46 (0.68 to 3.13)
rs560426	*ABCA4*	78:59	0.10	1.32 (0.94 to 1.85)	18:18	1.00	1.00 (0.52 to 1.92)
rs481931	*ABCA4*	28:25	0.68	1.12 (0.65 to 1.92)	3:8	0.13	0.38 (0.10 to 1.41)
rs4147811	*ABCA4*	26:25	0.89	1.04 (0.60 to 1.80)	5:10	0.20	0.50 (0.17 to 1.46)
rs138751793	*ARHGAP29*	5:7	0.56	0.71 (0.23 to 2.25)	1:2	0.56	0.50 (0.05 to 5.51)
rs6677101	*SLC25A24*	65:75	0.40	0.87 (0.62 to 1.21)	21:14	0.24	1.50 (0.76 to 2.95)
rs861020	*IRF6*	35:29	0.45	1.21 (0.74 to 1.97)	3:7	0.21	0.43 (0.11 to 1.66)
rs34743335	*IRF6*	4:2	0.41	2.00 (0.37 to 10.92)	0:0	NA	NA (NA)
rs642961	*IRF6*	29:29	1.00	1.00 (0.60 to 1.67)	2:7	0.10	0.29 (0.06 to 1.38)
rs7590268	*THADA*	49:48	0.92	1.02 (0.69 to 1.52)	8:8	1.00	1.00 (0.38 to 2.66)
rs4332945	*DYSF*	43:40	0.74	1.08 (0.70 to 1.65)	11:8	0.49	1.38 (0.55 to 3.42)
rs2303596	*DYSF*	45:57	0.23	0.79 (0.53 to 1.18)	12:8	0.37	1.50 (0.61 to 3.67)
rs227782	*DYSF*	73:65	0.50	1.12 (0.80 to 1.57)	20:13	0.22	1.54 (0.77 to 3.09)
rs115200552	*MSX1*	10:13	0.53	0.77 (0.34 to 1.75)	7:2	0.10	3.50 (0.72 to 16.85)
rs12532	*MSX1*	77:71	0.62	1.09 (0.79 to 1.50)	20:22	0.76	0.91 (0.50 to 1.67)
rs2674394	Gene desert	40:44	0.66	0.91 (0.59 to 1.40)	9:9	1.00	1.00 (0.40 to 2.52)
rs651333	*TULP4*	56:59	0.78	0.95 (0.66 to 1.37)	21:16	0.41	1.31 (0.68 to 2.52)
rs6558002	*EPHX2*	47:40	0.45	1.18 (0.77 to 1.79)	13:12	0.84	1.08 (0.49 to 2.37)
rs987525	8q24	71:59	0.29	1.20 (0.85 to 1.70)	19:20	0.87	0.95 (0.51 to 1.78)
rs894673	*FOXE1*	60:67	0.53	0.90 (0.63 to 1.29)	16:15	0.86	1.07 (0.53 to 2.16)
rs3758249	*FOXE1*	59:66	0.53	0.89 (0.63 to 1.27)	16:15	0.86	1.07 (0.53 to 2.16)
rs7078160	*VAX1*	60:44	0.12	1.36 (0.92 to 2.01)	18:10	0.13	1.80 (0.83 to 3.90)
rs4752028	*VAX1*	73:76	0.81	0.96 (0.70 to 1.32)	**27:13**	**0.03**[a]	**2.08 (1.07 to 4.03)**
rs10785430	*ADAMTS20*	61:59	0.86	1.03 (0.72 to 1.48)	15:11	0.43	1.36 (0.63 to 2.97)
rs9574565	*SPRY2*	69:55	0.21	1.26 (0.88 to 1.79)	18:17	0.87	1.06 (0.55 to 2.05)
rs8001641	*SPRY2*	22:22	1.00	1.00 (0.55 to 1.81)	9:6	0.44	1.50 (0.53 to 4.21)
rs17563	*BMP4*	44:44	1.00	1.00 (0.66 to 1.52)	10:15	0.32	0.67 (0.30 to 1.48)
rs1258763	*GREM1*	73:58	0.19	1.26 (0.89 to 1.78)	19:21	0.75	0.90 (0.49 to 1.68)
rs8049367	*ADCY9*	67:67	1.00	1.00 (0.71 to 1.40)	12:13	0.84	0.92 (0.42 to 2.02)
rs16260	*CDH1*	31:28	0.70	1.11 (0.66 to 1.85)	6:13	0.11	0.46 (0.18 to 1.21)
rs11642413	*CDH1*	62:49	0.22	1.27 (0.87 to 1.84)	14:11	0.55	1.27 (0.58 to 2.80)
rs1546124	*CRISPLD2*	53:44	0.36	1.21 (0.81 to 1.80)	9:14	0.30	0.64 (0.28 to 1.49)
rs4783099	*CRISPLD2*	75:64	0.35	1.17 (0.84 to 1.64)	15:21	0.32	0.71 (0.37 to 1.39)
rs8069536	*NTN1*	67:70	0.80	0.96 (0.68 to 1.34)	14:13	0.85	1.08 (0.51 to 2.29)
rs8081823	*NTN1*	58:56	0.85	1.04 (0.72 to 1.50)	14:15	0.85	0.93 (0.45 to 1.93)
rs17760296	*NOG1*	7:8	0.80	0.88 (0.32 to 2.41)	2:0	0.16	NA (NA)
rs227731	*NOG1*	47:49	0.84	0.96 (0.64 to 1.43)	20:11	0.11	1.82 (0.87 to 3.80)
rs7224837	*AXIN2*	19:27	0.24	0.70 (0.39 to 1.27)	1:6	0.06	0.17 (0.02 to 1.38)
rs3923086	*AXIN2*	2:3	0.65	0.67 (0.11 to 3.99)	1:0	0.32	NA (NA)
rs17820943	*MAFB*	49:42	0.46	1.17 (0.77 to 1.76)	15:12	0.56	1.25 (0.59 to 2.67)
rs13041247	*MAFB*	49:43	0.53	1.14 (0.76 to 1.72)	15:12	0.56	1.25 (0.59 to 2.67)
rs11696257	*MAFB*	48:43	0.60	1.12 (0.74 to 1.69)	14:12	0.69	1.17 (0.54 to 2.52)

Part B: Transmission Disequilibrium Test Subphenotype Analyses for NSCL/P

		NSCL			NSCLP		
rs1801131	*MTHFR*	**9:20**	**0.04**[a]	**0.45 (0.20 to 0.99)**	18:14	0.48	1.29 (0.64 to 2.59)
rs1801133	*MTHFR*	7:8	0.80	0.88 (0.31 to 2.41)	15:15	1.00	1.00 (0.49 to 2.05)
rs766325	*PAX7*	18:24	0.35	0.75 (0.41 to 1.38)	25:28	0.68	0.89 (0.52 to 1.53)
rs742071	*PAX7*	**50:30**	**0.03**[a]	**1.67 (1.06 to 2.62)**	32:45	0.14	0.71 (0.45 to 1.12)
rs560426	*ABCA4*	32:35	0.71	0.91 (0.57 to 1.48)	**46:24**	**8.55E-03**[a]	**1.92 (1.17 to 3.14)**
rs481931	*ABCA4*	10:13	0.53	0.77 (0.34 to 1.75)	18:12	0.27	1.50 (0.72 to 3.14)
rs4147811	*ABCA4*	8:10	0.64	0.80 (0.32 to 2.03)	18:15	0.60	1.20 (0.60 to 2.38)
rs138751793	*ARHGAP29*	1:2	0.56	0.50 (0.05 to 5.51)	4:5	0.74	0.80 (0.21 to 2.98)
rs6677101	*SLC25A24*	26:41	0.07	0.63 (0.39 to 1.04)	39:34	0.56	1.15 (0.72 to 1.82)
rs861020	*IRF6*	20:14	0.30	1.43 (0.72 to 2.83)	15:15	1.00	1.00 (0.49 to 2.05)
rs34743335	*IRF6*	2:1	0.56	2.00 (0.18 to 22.06)	2:1	0.56	2.00 (0.18 to 22.06)
rs642961	*IRF6*	16:15	0.86	1.07 (0.53 to 2.16)	13:14	0.85	0.93 (0.44 to 1.98)

(continued)

Table 3. (continued)

Part B: Transmission Disequilibrium Test Subphenotype Analyses for NSCL/P							
		NSCL			NSCLP		
SNP	Probable Gene/Loci	T:NT	*P*	OR (95% CI)	T:NT	*P*	OR (95% CI)
rs7590268	*THADA*	21:32	0.13	0.66 (0.38 to 1.14)	28:16	0.07	1.75 (0.95 to 3.23)
rs4332945	*DYSF*	21:17	0.52	1.24 (0.65 to 2.34)	22:23	0.88	0.96 (0.53 to 1.72)
rs2303596	*DYSF*	18:22	0.53	0.82 (0.44 to 1.53)	27:35	0.31	0.77 (0.47 to 1.27)
rs227782	*DYSF*	33:28	0.52	1.18 (0.71 to 1.95)	40:37	0.73	1.08 (0.69 to 1.69)
rs115200552	*MSX1*	6:3	0.32	2.00 (0.50 to 8.00)	4:10	0.11	0.40 (0.13 to 1.28)
rs12532	*MSX1*	39:32	0.41	1.22 (0.76 to 1.95)	38:39	0.91	0.97 (0.62 to 1.52)
rs2674394	Gene desert	21:17	0.52	1.24 (0.65 to 2.34)	19:27	0.24	0.70 (0.39 to 1.27)
rs651333	*TULP4*	26:26	1.00	1.00 (0.58 to 1.72)	30:33	0.71	0.91 (0.55 to 1.49)
rs6558002	*EPHX2*	15:18	0.60	0.83 (0.42 to 1.65)	32:22	0.17	1.46 (0.85 to 2.50)
rs987525	8q24	35:28	0.38	1.25 (0.76 to 2.06)	36:31	0.54	1.16 (0.72 to 1.88)
rs894673	*FOXE1*	27:31	0.60	0.87 (0.52 to 1.46)	33:36	0.72	0.92 (0.57 to 1.47)
rs3758249	*FOXE1*	27:31	0.60	0.87 (0.52 to 1.46)	32:35	0.71	0.91 (0.57 to 1.48)
rs7078160	*VAX1*	37:23	0.07	1.61 (0.96 to 2.71)	23:21	0.76	1.10 (0.61 to 1.98)
rs4752028	*VAX1*	32:38	0.47	0.84 (0.53 to 1.35)	41:38	0.74	1.08 (0.69 to 1.68)
rs10785430	*ADAMTS20*	25:28	0.68	0.89 (0.52 to 1.53)	36:31	0.54	1.16 (0.72 to 1.88)
rs9574565	*SPRY2*	35:29	0.45	1.21 (0.74 to 1.97)	34:26	0.30	1.31 (0.78 to 2.18)
rs8001641	*SPRY2*	12:12	1.00	1.00 (0.45 to 2.27)	10:10	1.00	1.00 (0.42 to 2.40)
rs17563	*BMP4*	22:16	0.33	1.38 (0.72 to 2.62)	22:28	0.40	0.79 (0.45 to 1.37)
rs1258763	*GREM1*	31:27	0.60	1.15 (0.69 to 1.92)	42:31	0.20	1.36 (0.85 to 2.16)
rs8049367	*ADCY9*	25:28	0.68	0.89 (0.52 to 1.53)	42:39	0.74	1.08 (0.70 to 1.67)
rs16260	*CDH1*	12:14	0.69	0.86 (0.40 to 1.85)	19:14	0.38	1.36 (0.68 to 2.71)
rs11642413	*CDH1*	25:22	0.66	1.14 (0.64 to 2.02)	37:27	0.21	1.37 (0.83 to 2.25)
rs1546124	*CRISPLD2*	25:22	0.66	1.14 (0.61 to 2.02)	28:22	0.40	1.27 (0.73 to 2.23)
rs4783099	*CRISPLD2*	39:35	0.64	1.11 (0.71 to 1.76)	36:29	0.39	1.24 (0.76 to 2.02)
rs8069536	*NTN1*	32:35	0.71	0.91 (0.57 to 1.48)	35:35	1.00	1.00 (0.63 to 1.60)
rs8081823	*NTN1*	30:20	0.16	1.50 (0.85 to 2.64)	28:36	0.32	0.78 (0.47 to 1.27)
rs17760296	*NOG1*	5:2	0.26	2.50 (0.49 to 12.89)	2:6	0.16	0.33 (0.07 to 1.65)
rs227731	*NOG1*	22:26	0.56	0.85 (0.48 to 1.49)	25:23	0.77	1.09 (0.62 to 1.92)
rs7224837	*AXIN2*	10:9	0.82	1.11 (0.45 to 2.73)	9:18	0.08	0.50 (0.22 to 1.11)
rs3923086	*AXIN2*	1:2	0.56	0.50 (0.05 to 5.51)	1:1	1.00	1.00 (0.06 to 15.99)
rs17820943	*MAFB*	18:22	0.53	0.82 (0.44 to 1.53)	31:20	0.12	1.55 (0.88 to 2.72)
rs13041247	*MAFB*	18:22	0.53	0.82 (0.44 to 1.53)	31:21	0.17	1.48 (0.85 to 2.57)
rs11696257	*MAFB*	18:22	0.53	0.82 (0.44 to 1.53)	30:21	0.21	1.43 (0.82 to 2.50)

95% CI, 95% confidence interval; NA, not applicable; NSCL, nonsyndromic cleft lip; NSCL/P, nonsyndromic cleft lip with or without cleft palate; NSCLP, nonsyndromic cleft lip and palate; NSCPO, nonsyndromic cleft palate only; NT, not transmitted; OR, odds ratio; SNP, single nucleotide polymorphism; T, transmitted.

[a]Loci that demonstrated overtransmission at threshold significance of $P \leq 0.05$ (in bold).

et al. 2015) and, to some extent, in European-derived populations (Estandia-Ortega et al. 2014; de Aguiar et al. 2015), though not all studies (Sozen et al. 2009) replicated the association. Interestingly, we have demonstrated in TDT analyses that *MTHFR* is significantly associated with NSCL among Africans and that it is the C minor allele of the A1298C (rs1801131) SNP that confers a reduced risk, suggesting that A is the risk allele. *AXIN2* has been implicated in the etiology of NSOFCs in multiple populations, except Africans, with rs3923086 demonstrating an association with NSCLP among Asians (Letra et al. 2012). Other studies (Mostowska et al. 2012; de Araujo et al. 2015) have replicated the association between *AXIN2* and NSCL/P. Here, we have demonstrated that rs3923086 (*AXIN2*) is also associated with NSCLP among Africans in DFAM analyses. Other candidate genes (e.g., *DYSF*) also showed evidence of association with NSOFCs among Africans, buttressing the relevance of this approach in etiologic "gene hunting."

Other SNPs, other than the already-reported ones, may be responsible for the associations between certain loci and NSOFCs in some ethnicities. Through direct DNA sequencing of the *MSX1* gene, we observed overtransmission of the minor allele of rs115200552 in NSOFC cases. Subsequent genotyping of this SNP in 3,585 individuals showed that this SNP was associated with NSCPO ($P = 0.01$) in case-control meta-analyses, although family-based studies suggest that this marker may be a risk allele for NSCLP. Earlier studies involving Africans from Nigeria implicated *MSX1* in the etiology of NSCL/P (Butali et al. 2011).

We could not detect a formal association between some GWASs and candidate gene loci and NSCL/P, presupposing that 1) these loci may not play a role in the etiology of NSCL/P in Africans or 2) the genotyped SNPs may not be the tag SNPs for Africans. Lack of statistical power due to sample size and low MAF of the genotyped SNPs in Africans could also be possible reasons. For example, rs2235371—an SNP of *IRF6*

Table 4. Family-Based Association for Disease Traits for Cases and Relatives.

		P Values			
SNP	Probable Gene/Loci	NSCL/P	NSCL	NSCLP	NSCPO
rs1801131	*MTHFR*	0.70	0.68	0.24	0.67
rs1801133	*MTHFR*	0.82	0.51	0.59	0.29
rs766325	*PAX7*	0.61	0.71	0.74	0.24
rs742071	*PAX7*	0.32	**0.02**[a]	0.29	0.96
rs560426	*ABCA4*	**2.59E-02**[a]	0.72	**4.75E-03**[a]	0.80
rs481931	*ABCA4*	0.15	0.55	0.16	0.61
rs4147811	*ABCA4*	0.29	0.44	0.48	0.51
rs138751793	*ARHGAP29*	0.38	0.66	0.43	0.40
rs6677101	*SLC25A24*	1.00	0.80	0.64	0.24
rs861020	*IRF6*	0.43	0.23	0.98	0.35
rs34743335	*IRF6*	0.32	0.52	0.47	0.61
rs642961	*IRF6*	0.83	0.99	0.98	0.15
rs11119388	*SYT14*	0.83	0.85	0.92	0.91
rs7590268	*THADA*	0.85	0.30	0.18	0.77
rs4332945	*DYSF*	**0.04**[a]	**0.02**[a]	0.60	0.62
rs2303596	*DYSF*	0.81	0.84	0.53	0.60
rs227782	*DYSF*	0.36	0.48	0.55	0.47
rs115200552	*MSX1*	0.89	0.13	**3.50E-02**[a]	0.08
rs12532	*MSX1*	0.67	0.96	0.30	0.43
rs2674394	Gene desert	0.59	0.11	0.58	0.51
rs651333	*TULP4*	0.92	0.90	0.63	0.20
rs6558002	*EPHX2*	0.38	0.77	0.27	0.52
rs987525	8q24	0.80	0.50	0.52	0.99
rs894673	*FOXE1*	0.69	0.88	0.46	0.55
rs3758249	*FOXE1*	0.69	0.86	0.46	0.55
rs7078160	*VAX1*	0.21	0.18	0.77	0.28
rs4752028	*VAX1*	0.88	0.44	0.30	0.06
rs10785430	*ADAMTS20*	0.84	0.86	0.62	0.66
rs9574565	*SPRY2*	0.07	0.16	0.28	0.22
rs8001641	*SPRY2*	0.32	0.19	0.88	0.64
rs375489721	*MIR17HG*	NA	NA	NA	NA
rs185831554	*MIR17HG*	0.32	0.32	NA	NA
rs17563	*BMP4*	0.66	0.15	0.80	0.70
rs1258763	*GREM1*	0.14	1.00	0.06	0.98
rs8049367	*ADCY9*	0.23	0.24	0.56	0.18
rs16260	*CDH1*	0.59	0.59	0.36	0.46
rs11642413	*CDH1*	0.33	0.81	0.08	0.88
rs1546124	*CRISPLD2*	0.30	0.53	0.45	0.15
rs4783099	*CRISPLD2*	0.17	0.14	0.89	0.37
rs8069536	*NTN1*	0.58	0.47	0.87	0.23
rs8081823	*NTN1*	0.97	0.30	0.19	0.89
rs17760296	*NOG1*	0.63	0.25	0.97	0.63
rs227731	*NOG1*	0.24	0.41	0.43	0.09
rs7224837	*AXIN2*	0.20	0.75	0.12	0.35
rs3923086	*AXIN2*	0.89	0.70	**2.88E-03**[a]	0.85
rs17820943	*MAFB*	0.31	0.88	0.14	0.65
rs13041247	*MAFB*	0.37	0.83	0.21	0.63
rs11696257	*MAFB*	0.46	0.89	0.26	0.77

NA, not applicable; NSCL, nonsyndromic cleft lip; NSCL/P, nonsyndromic cleft lip with or without cleft palate; NSCLP, nonsyndromic cleft lip and palate; NSCPO, nonsyndromic cleft palate only; SNP, single-nucleotide polymorphism.
[a]Loci that demonstrated overtransmission at threshold significance (in bold).

that is in high-linkage disequilibrium and the same locus as rs642961 and that has been associated with NSCL/P among mostly Asians (Sun et al. 2015) and in some Europeans (Zucchero et al. 2004)—does not exist in the African population (http://browser.1000genomes.org/index.html). It is also possible that even when no associations are detected between reported loci and NSOFCs, potentially pathogenic variants may be observed in NSOFC cases. Therefore, GWASs and whole genome sequencing of NSOFC cases from Africa are required to detect more risk loci.

Subphenotype and subpopulation analyses (even among the same racial group) may be crucial in detecting an association

Table 5. Novel, Rare, and Potentially Etiologic Variants Observed in Sequenced Genes.

Part A: Variants Observed in Cases and Some Parents but Not in Controls				
HGVS	HGVp	Total No. of Cases with Variant	Subphenotype of Cases with Variant	Segregation Analyses
			ARHGAP29	
c.341-30T>A	NA	1	NSCL	NA
c.511-107T>C	NA	2	NSCLP and NSCPO	NA
c.967A>G	p.Asn323Asp	1	NSCL	Absent in father
c.1277delAinsTA	p.Lys426IlefsTer6	1	NSCLP	Absent in mother
c.1281+4A>G	NA	1	NSCLP	Observed in clinically unaffected mother
			PAX7	
c.1227G>A	p.Leu409Leu	1	NSCL	NA

Part B: Bioinformatics-Predicted Effects of Potentially Pathogenic Variants				
HGVS	Polyphen-2	SIFT	Human Splice Finder	RegulomeDB
			ARHGAP29	
c.341-30T>A	NA	NA	Alteration of ESS site	NA
c.511-107T>C	NA	NA	Alteration of ESS site and creation of new ESE site	NA
c.967A>G	Benign	Deleterious	NA	NA
c.1277delAinsTA	NA	NA	NA	NA
c.1281+4A>G	NA	NA	Alteration of wildtype donor site	NA
			PAX7	
c.1227G>A	Benign	Tolerated	Alteration of an ESE site	NA

All analyses were based on genome assembly number GRCh37/hg19, 2009 (http://genome.ucsc.edu).
ESE, exonic splicing enhancer; ESS, exonic splicing silencer; NA, not applicable; NSCLP, nonsyndromic cleft lip and palate; NSCL, nonsyndromic cleft lip only; NSCPO, nonsyndromic cleft palate only.

between certain loci and NSOFCs. In both TDT and DFAM analyses, we observed that rs560426 of *ABCA4* was associated with NSCLP but not the other OFC subphenotypes. Case-control analyses further suggested that the *ABCA4* locus may be crucial in NSOFC etiology in all 3 African populations. *PAX7* (rs742071) exhibited nominal association with NSCL/P in case-control meta-analyses, with subpopulation analyses suggesting that this signal originated mainly from the Ethiopian and Nigerian cohorts that exhibited some level of heterogeneity. However, TDT and DFAM subphenotype analyses demonstrated that rs742071 exhibited overtransmission in NSCL cases in all 3 populations. In case-control meta-analyses, *VAX1* (rs7078160) was nominally associated with NSCL/P, with subpopulation analyses suggesting the 2 West African countries (largely Ghana) drive this signal.

Rare variants, but not necessarily common variants, may account for the link between certain loci and NSOFCs. We observed many missense mutations and 1 frameshift mutation in sequenced genes. No de novo occurrence was observed for any of these variants due to the unavailability of some parental samples. Moreover, some of the novel variants were also observed in clinically unaffected parents and controls. We sequenced the novel variants in 96 controls from Ghana, and the likelihood of identifying these novel variants in more controls (i.e., >96) is possible. Nonetheless, these variants are absent in >1,000 individuals in the 1000 Genomes database (with >300 Africans), >61,000 individuals in the ExAC database, as well as 6,500 individuals in the EVS database. There is also the need to functionally validate the pathogenicity or otherwise of these variants in vivo. Rare variants in *ARHGAP29* (Leslie et al. 2012), *PAX7* and *VAX1* (Butali et al. 2013; Leslie et al. 2015), *BMP4* (Suzuki et al. 2009), *FOXE1* (Moreno et al. 2009), *MAFB* (Butali, Mossey, et al. 2014), and *MSX1* (Liang et al. 2012) have been observed in NSOFC cases.

The incidence of OFC in Africans is much lower than in Europeans and Asians (Mossey and Modell 2012; Butali, Adeyemo, et al. 2014), even though these populations may share the same or similar genetic susceptibility loci for OFCs, as observed in the present study. Although underascertainment due to a lack of birth defect registries in most African countries could be a contributing factor (Butali, Adeyemo, et al. 2014), the low incidence of OFCs among Africans may be real, as African-derived populations in the Caribbean have a low OFC incidence similar to that of their ancestral population (Mossey and Modell 2012). We therefore hypothesize the possible existence of genetic protective variants in the African genome, whose "rescue mission" reduces clefting. The identification and elucidation of such protective variants can be translated to European and Asian populations to bring about reduced OFC incidence and eventually prevention.

Conclusion

The present study has shown evidence of an association of certain loci with NSOFCs at both nominal and threshold significance. For instance, we have for the first time shown that the

8q.24 locus is a risk locus in Africans. Our study has thus corroborated an earlier suggestion that the 8q24 locus may be a risk locus for NSCL/P across major ethnicities, although the effect size is smaller in Asians due to a lower MAF. Subphenotype as well as subpopulation analyses and genotyping of other SNPs, other than those already reported for some loci, may be crucial in identifying NSOFC loci in various ethnicities and populations. We have also demonstrated the existence of rare variants, both novel and known, in NSOFC cases from Africa. In conclusion, we have for the first time demonstrated associations between the SNPs that we studied and NSOFC among Africans. Our study is crucial for understanding the genetic architecture of NSOFCs in Africans and further suggests the need to carry out GWASs and whole genome sequencing for every ethnicity as far as complex traits are concerned.

Author Contributions

L.J.J. Gowans, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; W.L. Adeyemo and M. Eshete, contributed to conception, design, and data acquisition, critically revised the manuscript; P.A. Mossey, contributed to conception, data acquisition, and analysis, critically revised the manuscript; T. Busch, contributed to design, data acquisition, and interpretation, critically revised the manuscript; B. Aregbesola, contributed to data acquisition and critically revised the manuscript; P. Donkor, contributed to design, data acquisition, and interpretation, critically revised the manuscript; F.K.N. Arthur, contributed to design, data acquisition, and analysis, critically revised the manuscript; S.A. Bello, contributed to data acquisition, critically revised the manuscript; A. Martinez, M. Li, and E. Augustine-Akpan, contributed to data acquisition and analysis, critically revised the manuscript; W. Deressa, contributed to data acquisition, critically revised the manuscript; P. Twumasi, contributed to design, critically revised the manuscript; J. Olutayo, M. Deribew, P. Agbenorku, A.A. Oti, R. Braimah, G. Plange-Rhule, M. Gesses, S. Obiri-Yeboah, G.O. Oseni, P.B. Olaitan, L. Abdur-Rahman, F. Abate, T. Hailu, P. Gravem, and M.O. Ogunlewe, contributed to data acquisition, critically revised the manuscript; C.J. Buxó, M.L. Marazita, and A.A. Adeyemo, contributed to data analysis and interpretation, critically revised the manuscript; J.C. Murray and A. Butali, contributed to conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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